1-1-2006

Two domains within the Mycoplasma hyopneumoniae cilium adhesin bind heparin

C. Jenkins
Elizabeth Macarthur Agricultural Institute

J. L. Wilton
Elizabeth Macarthur Agricultural Institute

F. C. Minion
Iowa State University, USA

L. Falconer
Elizabeth Macarthur Agricultural Institute

Mark J. Walker
University of Wollongong, mwalker@uow.edu.au

See next page for additional authors

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Abstract

Mycoplasma hyopneumoniae is the causative agent of porcine enzootic pneumonia, a chronic and economically significant respiratory disease that affects swine production worldwide. M. hyopneumoniae adheres to, and adversely affects the function of ciliated epithelial cells of the respiratory tract, and the cilium adhesin (P97), is intricately but not exclusively involved in this process. Although binding of pathogenic bacteria to glycosaminoglycans is a recognised step in pathogenesis, knowledge of glycosaminoglycan-binding proteins is lacking in M. hyopneumoniae. However, heparin and other sulfated polysaccharides are known to block the binding of M. hyopneumoniae to purified swine respiratory cilia. In this study, four regions within the cilium adhesin were examined for their ability to bind heparin. Cilium adhesin fragments comprising 653 amino acids of the N- terminus and 301 amino acids of the C-terminus (containing two repeat regions, R1 and R2) were cloned and expressed. These fragments bound heparin in a dose-dependent and saturable manner with physiologically significant binding affinities of 0.27±0.02 μM and 1.89±0.33 μM, respectively. Heparin binding of both fragments was strongly inhibited by the sulfated polysaccharides fucoidan and mucin but not by chondroitin sulfate B. When the C-terminal repeat regions R1 and R2 were cloned separately and expressed, heparin-binding activity was lost, suggesting that both regions are required for heparin binding. The ability of the cilium adhesin to bind heparin indicates that this molecule plays a multi-functional role in the adherence of M. hyopneumoniae to host respiratory surfaces and therefore has important implications with respect to the pathogenesis of this organism.

Keywords

Mycoplasma, hyopneumoniae, heparin

Publication Details

This article was originally published as Jenkins, C, Wilton, JL, Minion, FC, Falconer, L, Walker, MJ and Djordjevic, SP, Two domains within the Mycoplasma hyopneumoniae cilium adhesin bind heparin, Infection and Immunity, 74(1), 2006, 481-487.

Authors

C. Jenkins, J. L. Wilton, F. C. Minion, L. Falconer, Mark J. Walker, and S. P. Djordjevic

Two domains within the *Mycoplasma hyopneumoniae* cilium adhesin bind heparin

Cheryl Jenkins[1,2], Jody L. Wilton[1], F. Chris Minion[3], Linda Falconer[1], Mark J. Walker[2] and Steven P. Djordjevic[1*]

[1]Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Camden, NSW 2570, Australia

[2]School of Biological Sciences, University of Wollongong, NSW, Australia

[3]Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa 50011

[*]**Corresponding Author**

Mailing address:

Microbiology and Immunology

PMB 8

Camden NSW 2570

Ph: +61 2 4640 6426

Fax: +61 2 4640 6438

E-mail: steve.djordjevic@agric.nsw.gov.au

Running title: The cilium adhesin is a heparin binding protein

Abstract

Mycoplasma hyopneumoniae is the causative agent of porcine enzootic pneumonia, a chronic and economically significant respiratory disease that affects swine production worldwide. *M. hyopneumoniae* adheres to, and adversely affects the function of ciliated epithelial cells of the respiratory tract, and the cilium adhesin (P97), is intricately but not exclusively involved in this process. Although binding of pathogenic bacteria to glycosaminoglycans is a recognised step in pathogenesis, knowledge of glycosaminoglycan-binding proteins is lacking in *M. hyopneumoniae*. However, heparin and other sulfated polysaccharides are known to block the binding of *M. hyopneumoniae* to purified swine respiratory cilia. In this study, four regions within the cilium adhesin were examined for their ability to bind heparin. Cilium adhesin fragments comprising 653 amino acids of the N- terminus and 301 amino acids of the C-terminus (containing two repeat regions, R1 and R2) were cloned and expressed. These fragments bound heparin in a dose-dependent and saturable manner with physiologically significant binding affinities of 0.27±0.02 μM and 1.89±0.33 μM, respectively. Heparin binding of both fragments was strongly inhibited by the sulfated polysaccharides fucoidan and mucin but not by chondroitin sulfate B. When the C-terminal repeat regions R1 and R2 were cloned separately and expressed, heparin-binding activity was lost, suggesting that both regions are required for heparin binding. The ability of the cilium adhesin to bind heparin indicates that this molecule plays a multi-functional role in the adherence of *M. hyopneumoniae* to host respiratory surfaces and therefore has important implications with respect to the pathogenesis of this organism.

Introduction

Mycoplasma hyopneumoniae is the aetiological agent of porcine enzootic pneumonia, a chronic respiratory disease that causes significant economic losses to the swine industry (30). Infections are established via colonization of porcine respiratory epithelia, a process initiated by the adherence of bacterial cells to host cilia. Successful colonization results in ciliostasis and shedding of cilia from the epithelial surface, thereby disrupting the muco-ciliary escalator and leaving the host susceptible to secondary infections (3, 9).

Studies have shown that the cilium adhesin, P97, is essential for the adherence of *M. hyopneumoniae* and the onset of disease (15, 37). The cilium adhesin gene encodes a 126 kDa pre-protein. The full length protein is not present in *in vitro* grown cells in detectable amounts due to post-translational processing. A major cleavage event at position 195 generates the functional adhesin (P97) and the N-terminal fragment (P22). P97 is further cleaved to yield a host of other peptides (10). While the functions of these peptides (apart from P97) are unknown, proteomic and immunogold studies demonstrate that they remain associated with the cell surface or the surrounding matrix (10).

Like several other characterized mycoplasma adhesins, P97 contains repetitive elements within the C-terminal portion of its amino acid sequence (4, 8, 15). P97 contains two such repeat regions, designated R1 and R2, with R1 (sequence AAKPV/E) being identified as the cilium binding epitope (16). Size variation in the cilium adhesin protein of different *M. hyopneumoniae* strains (95-97 kDa) has been accounted for by differences in the number of copies of R1, which varies from 9 in the non-adherent J strain, to 15 in the pathogenic 232 strain (35). A minimum of 8 copies of the R1 repeat are necessary for cilial binding, but this is not sufficient to confer the ability to adhere *in vivo* (16, 23).

These data suggest that there are additional proteins and mechanisms involved in adherence to host epithelia.

The importance of binding to host extracellular matrix (ECM) components in initiating pathogenic processes has been well documented. By binding to ECM proteins such as fibronectin, vitronectin, laminin, and collagen, pathogenic microbes are able to mediate interactions with host cells (27), and in some cases enhance their invasive potential (12, 18, 28). Furthermore, it has been demonstrated that many pathogens are able to recruit exogenous glycosaminoglycans (eg. heparin, heparan sulfate and chondroitin sulfate B) to their surfaces to facilitate interactions between the pathogen and host ECM components, which are themselves glycosaminoglycan binding proteins (13, 21, 34). Binding to glycosaminoglycans is also believed to enable pathogenic bacteria to evade the host immune system (7, 12) and modulate the inflammatory response (13) by allowing bridging interactions with host chemokines and cytokines. Thus, glycosaminoglycan-binding proteins are considered to be of central importance to the infection process.

Previous experiments have revealed that adherence of purified recombinant P97 to cilia is inhibited by sulfated glycosaminoglycans (15). These findings suggest a possible interaction between the P97 protein and sulfated glycosaminoglycans which may act as receptor analogs for the *M. hyopneumoniae* adhesin (15, 36, 38); however; these studies did not directly demonstrate such an interaction. Furthermore, the domains within the cilium adhesin responsible for this interaction were not identified. In this study we identify regions within the cilium adhesin that interact with the glycosaminoglycan,

heparin. The ability of *M. hyopneumoniae* cell surface molecules to bind heparin may play a significant role in the pathogenesis of this organism.

Materials and Methods

Bacterial strains and plasmids. *M. hyopneumoniae* strain J (NCTC 10110) was cultivated to mid-log phase (medium pH range 6.8-7.2) in modified Friis broth at 37°C on a rotary shaker as described previously (33). The *Escherichia coli* plasmid expression and maintenance strains were M15 (pREP4) and JM109 respectively, and were grown in Luria-Bertani (LB) medium (31) at 37°C. Broth cultures were incubated on a rotary shaker at 200-250 rpm to achieve aeration. For selection of transformants, LB medium was supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin as required.

DNA extraction. Chromosomal DNA was extracted from the J strain of *M. hyopneumoniae* using a phenol/chloroform method followed by dialysis against TE buffer as described previously (11). Plasmid DNA was extracted from *E. coli* using either the Qiagen mini-prep or midi-prep kit according to the manufacturer's instructions (Qiagen, Alameda, Calif.).

Plasmid constructs. Adhesin molecules from two different strains of *M. hyopneumoniae* were used to generate poly-histidine fusion proteins of different regions of the coding sequence. Because UGA codons encode tryptophan in mycoplasmas, they cause translation problems in *E. coli*, resulting in premature stops during expression of cloned sequences. To circumvent this problem, three clones were generated by amplifying chromosomal DNA from strain J, with PCR primers designed to avoid in-frame TGA codons. An additional construct was generated using a clone containing a

large fragment of the P97 sequence from strain P5722 (19) in which the TGA codons had been mutagenized to TGGs. This approach was deemed valid because the sequence differences between these two P97 homologs is less than 4%, with most of the differences accounted for by variation in the number of R1 and R2 repeats.

The first construct contained the region coding for the N-terminus of the cilium adhesin (amino acids 106-758 of mutagenized gene sequence) and was referred to as $F1_{P97}$. The PCR template for this construct was pISM405 in which the TGA codons had been converted to TGGs (10). The second construct, referred to as $F2_{P97}$ (amino acids 768-1069 of J strain sequence), contained the cilium adhesin repeat domains coding for both R1, the cilium binding epitope, and R2. This gene fragment was further subcloned to yield two additional constructs referred to as $F3_{P97}$ (amino acids 768-914 of J strain sequence) and $F4_{P97}$ (amino acids 935-1069 of J strain sequence), which contained the R1 and R2 regions, respectively (Fig. 1).

To clone specific sequences, gene fragments were amplified via PCR using *Taq* polymerase (Qiagen). Primers used for the amplification of the gene fragments encoding $F1_{P97}$, $F3_{P97}$ and $F4_{P97}$ were as follows: F1F (5'-GGGGATCCCAAGATCCTGAATAT ACC) and F1R (5'- GGCTGCAGTTAGGCTGCTTTAAGGAAAAATGC) (underlined sequences represent BamHI and PstI restriction sites, respectively); F3F (5'- GGGT CGACAAATTAGACGATAATCTTCAG) and F3R (5'- GGCTGCAGTTAC TCGCTTTGATGAACTAGTTC) (underlined sequences represent SalI and PstI restriction sites, respectively); F4F (5'- GGGGATCCCAGGAAGTCAAGGTAACT AGT) and F4R (5'- GGCTGCAGCCCGGGTTAGGATCACCGGATTTTGAATC) (underlined sequences represent BamHI and PstI restriction sites, respectively). Primers

F3F and F4R were used to amplify the gene fragment corresponding to $F2_{P97}$. The $F1_{P97}$-$F4_{P97}$ cilium adhesin fragments were digested with the appropriate restriction enzymes and ligated into linearized pQE-9 with 1 U of T4 DNA ligase (Roche, Basel, Switzerland) at 10°C overnight. Ligated plasmids were electroporated into *E. coli* M15 (pREP4) cells using a BioRad Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) at 2.5 kV, 25 μFD and 200 Ω according to the manufacturer's instructions. All plasmid constructs were confirmed via DNA sequencing.

Protein expression and purification. All cilium adhesin fragments were expressed as hexahistidyl fusion proteins and purified by nickel affinity chromatography. Briefly, *E. coli* M15 (pREP4) cells were grown to mid-log phase and induced with a final concentration of isopropyl-β-D-thiogalactopyranoside of 1 mM. Cells were harvested by centrifugation (4000 x *g* for 20 min) and then lysed in 8 M urea buffer (8 M urea, 0.01 M Tris, 0.1 M NaH_2PO_4; pH 8.0) with gentle rocking for 1 h. Cell debris was pelleted by centrifugation at 10,000 x *g* for 30 min. To the supernatant, 0.25 volumes of 50% Ni-NTA slurry (Qiagen) was added and the solution allowed to mix gently for 1 h. The solution was loaded onto a glass column and washed twice with 8M urea buffer (pH 6.3) to remove unbound protein. Bound protein was eluted with low pH urea buffers (pH 5.9 and pH 4.5). Proteins were analysed for purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then dialysed for 48 h with multiple buffer changes against phosphate buffered saline (PBS; 10 mM sodium phosphate – 150 mM sodium chloride, pH 7.4)-0.1% SDS or PBS-5% glycerol to remove the urea. The concentration of the purified proteins was estimated using the Bradford assay (Bio-Rad Laboratories).

Production of polyclonal antisera. Antisera to the $F1_{P97}$-$F4_{P97}$ adhesin fragments were generated via primary and secondary intramuscular injections of antigen into New Zealand white rabbits at two-week intervals. Antigens (approximately 0.5 mg) were prepared for injection by mixing equal volumes of purified protein (approximately 500 µl) and Freund's Incomplete Adjuvant (Sigma Aldrich, St. Louis, Missouri). Pre-immune serum was collected prior to the primary injection and served as control antiserum. A trial bleed was performed 10-14 days after the secondary injection of antigen and immune responses to the antigen at this stage were assessed via immunoblotting. Positive sera were collected via cardiac bleed during which the rabbits were anaesthetized and euthanized as previously described (32).

Heparin binding assays. For ligand dot blot assays, purified proteins were diluted to 5 µg/ml in PBS. To denature samples, proteins were diluted in electrophoresis sample buffer (60 mM Tris pH 6.8, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and boiled for 5 min. A 100 µl volume of each sample was spotted onto a Hybond-C super nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) that had been pre-wetted in Tris-buffered saline (TBS; 10 mM Tris, 150 mM sodium chloride; pH 7.4) using a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories). The membrane was blocked with 300 µl per well of blocking solution (1% bovine serum albumin (BSA) in TBS) and then washed twice under vacuum with 300 µl per well of TTBS wash solution (0.05% Tween 20 in TBS). The membrane was removed from the manifold and placed in a dish containing 100 µg/ml biotinylated heparin (Sigma) diluted in 1% BSA-TTBS. The membrane was incubated for 1 h with gentle rocking and was then washed 3 x 10 min in TTBS. Streptavidin peroxidase (Roche) was

diluted 1:3000 in 1% BSA-TTBS and added to the membrane. After 1 h incubation the blot was washed 3 x 10 min with TTBS. The membrane was equilibrated with 100 mM Tris (pH 7.6) and developed with 3,3'-diaminobenzidine (Sigma) solution.

For microtitre plate binding assays, 96 well plates (Linbro/Titertek; ICN Biomedicals Inc., Aurora, Ohio) were coated overnight in a humidified chamber with 0.5 µg per well purified protein diluted in 100 µl carbonate coating buffer (18 mM $NaHCO_3$, 27 mM Na_2CO_3; pH 9.5). Following overnight incubation, the plates were washed 5 times with PBS-0.2% Tween 20 (200 µl wash solution per well) using an automatic 96PW plate washer (SLT Labinstruments, Crailsheim, Germany). The wells were blocked with 100 µl of blocking solution (2% skim milk in PBS) for 1 h in a humidified chamber. The plates were washed 5 times and biotinylated heparin which had been pre-diluted in 1% skim milk-PBS was added in increasing concentrations to the wells. Final concentrations of biotinylated heparin were 0, 1, 5, 10, 20, 50, and 100 µg/ml. Aliquots of 100 µl of each concentration were added to microtitre plate wells; the plates were then incubated for 1 h. After washing the plates as above, 100 µl of streptavidin-peroxidase (Roche) diluted 1:3000 in 1% skim milk-PBS was added to each well and incubated for 1 h. The plates were washed and developed using 1 mM 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) substrate (Sigma) in citrate buffer (100 mM citric acid, 200 mM Na_2HPO_4, pH 4.2) which had been activated with hydrogen peroxide. Plates were developed with shaking on a Titramax 1000 microtitre plate shaker (Heidolph, Schwabach, Germany), and the optical density at 414 nm measured with a Multiskan Ascent plate reader (Thermo Labsystems, Franklin, Mass.) at 7, 15, 25 and 45 min intervals.

Microtitre plate assays for determining the specificity of heparin binding, were performed as above but with serial 2-fold dilutions of biotinylated heparin, beginning at saturating concentration (100 µg/ml for $F1_{P97}$ and 50 µg/ml for $F2_{P97}$), each of which were pre-mixed with a 50-fold excess of unlabelled heparin prior to addition to the plates.

Competitive binding assays were also performed as above but with the addition of a 1, 5, 10, 20, 30, 40 and 50-fold excess of a range of sulfated polysaccharides. Inhibitors were pre-mixed with biotinylated heparin immediately prior to addition to the wells and included unlabelled heparin, fucoidan, mucin and chondroitin sulfate B (Sigma). In all microtitre plate assays, controls were performed in uncoated wells, wells where no heparin was added, or wells where no streptavidin-peroxidase was added. Controls which employed protein-specific antiserum and horseradish peroxidase (HRP)-labelled anti-rabbit IgG (Chemicon, Temecula, Calif.) were also conducted to ensure that proteins were adhering to the microtitre plate wells. All experiments were performed in triplicate. Results were graphed with GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, Calif.; www.graphpad.com) using non-linear regression.

Results

Bioinformatic identification of putative heparin binding motifs. The cilium adhesin was examined for potential heparin-binding sites within its amino acid sequence using established criteria (5, 6). In many instances, glycosaminoglycan binding proteins are reported to contain consensus sequences of two types: XBBXBX and XBBBXXBX, where B represents a basic residue and X any other amino acid. The $F1_{P97}$ and $F2_{P97}$/$F3_{P97}$ domains of the cilium adhesin each contain one motif that matches the former consensus

sequence (DKKDKS and DKKVKE respectively) (Fig. 2). Several heparin binding proteins have been identified that do not contain the consensus sequences but possess sequences that are relatively rich in basic residues (20, 29). Both $F1_{P97}$ and $F2_{P97}$ also contain relatively large numbers of basic amino acids across the length of their sequences (Fig. 2).

Heparin binding assays. To determine whether the cilium adhesin fragments bound to heparin and to assess the importance of their protein conformation in this process, ligand blots were performed. Standard ligand blots involving the transfer of proteins from denaturing polyacrylamide gels to PVDF gave only weak or unreliable results (data not shown). In a dot blot assay, strong and reproducible reactions with biotinylated heparin were observed for undenatured $F1_{P97}$ and $F2_{P97}$, while a weak reaction was observed for undenatured $F4_{P97}$ (Fig. 3). Interestingly, the same protein fragments did not show a strong reaction with heparin when they were denatured prior to blotting, indicating that protein conformation may play a role in the interaction with heparin.

Quantification of heparin binding by the cilium adhesin domains and determination of binding affinities was performed by a microtiter plate binding assay. The recombinant cilium adhesin fragments $F1_{P97}$-$F4_{P97}$ were shown to reliably bind to microtitre plates by interacting with fragment-specific antibodies (data not shown). Experiments were then performed with increasing amounts of biotinylated heparin up to a saturating concentration. Fig. 4 indicates the total binding (specific and non-specific) of the cilium adhesin fragments to heparin. Both $F1_{P97}$ and $F2_{P97}$ were found to bind heparin in a dose-dependent and saturable manner. In contrast, $F3_{P97}$ and $F4_{P97}$ showed low-level, non-saturable binding, indicating a non-specific interaction.

To determine whether the binding of $F1_{P97}$ and $F2_{P97}$ to heparin was the result of a specific interaction, binding assays were undertaken in which biotinylated heparin was added in the presence and absence of a 50-fold concentration of unlabelled heparin. Binding of heparin to both $F1_{P97}$ and $F2_{P97}$ was shown to be highly specific with minimal non-specific binding observed (Fig. 5A and 5B). Affinity constants were determined using the specific binding data (total binding minus non-specific binding) and were found to be 0.27±0.02 μM and 1.89±0.33 μM for $F1_{P97}$ and $F2_{P97}$, respectively. These values are comparable to those derived from replicate experiments.

Inhibition of heparin binding using sulfated polysaccharides. The nature of the interaction between the cilium adhesin fragments and heparin was further characterised in competitive binding experiments using the sulfated polysaccharides heparin, fucoidan, mucin and chondroitin sulfate B. For both $F1_{P97}$ and $F2_{P97}$, biotinylated heparin binding was significantly inhibited by heparin. Furthermore, for both $F1_{P97}$ and $F2_{P97}$, fucoidan and mucin were shown to be strong competitors while chondroitin sulfate B did not inhibit the interaction of heparin with either adhesin domain (Fig. 6A and 6B). Notably, both fucoidan and mucin gave stronger inhibition than unlabelled heparin. These results suggest that both $F1_{P97}$ and $F2_{P97}$ are able to bind fucoidan and mucin in addition to heparin.

Discussion

In order to establish infection, bacterial pathogens must first adhere to and colonize host epithelia. For many bacteria, the initial site of contact with host tissues is at the level

of the ECM, therefore the ability to bind components of the ECM is widespread amongst pathogenic microbes (12, 13, 22, 34). Because of their fastidious nature and obligate parasitic mode of life, mycoplasmas require particularly efficient mechanisms for adherence. For *M. hyopneumoniae*, knowledge about the mechanisms of adhesion to host tissues is currently confined to the cilium adhesin and its interaction with the cilia of the porcine respiratory tract (15, 17, 23, 37). No ECM-binding proteins have been identified in this organism; however, previous studies indicated that binding of the cilium adhesin to cilia could be inhibited by heparin, providing indirect evidence that the adhesin is able to bind this molecule (15, 38).

In this study, two non-overlapping regions of the *M. hyopneumoniae* cilium adhesin, $F1_{P97}$ and $F2_{P97}$, were shown to bind heparin in a dose-dependent, saturable manner. These results indicate that there are at least two distinct heparin-binding domains within the adhesin protein. Both of these regions contain clusters of basic amino acids that match the consensus for glycosaminoglycan-binding motifs (6). One of these protein regions, $F2_{P97}$, contains both of the cilium adhesin repeat regions, R1 and R2. The heparin-binding consensus motif of $F2_{P97}$ lies within the $F3_{P97}$ region (Fig. 1A), which would suggest that $F3_{P97}$ should also bind heparin. As $F3_{P97}$ was not shown to bind heparin in either the ligand blot or microtitre plate assay, it must be concluded that the heparin binding motif in $F2_{P97}$ alone was not sufficient to confer binding activity, indicating that additional sequences are required. $F4_{P97}$ also failed to show any significant interaction with heparin in microtitre plate assays indicating that while R1 and R2 are unable to bind heparin independently, the intact $F2_{P97}$ fragment (containing both of these regions) displays heparin-binding activity. The fact that both of the cilium adhesin repeat

regions are required for heparin binding suggests that this interaction is conformation-dependent rather than due to the presence of a specific linear amino acid motif. Furthermore, the idea that protein conformation plays a role in heparin-binding is supported by the ligand blot data which demonstrated that undenatured $F1_{P97}$ and $F2_{P97}$ were able to bind heparin while the denatured forms were unable to do so.

Alternatively, since 20 amino acids (914-935) were omitted during the construction of $F3_{P97}$ and $F4_{P97}$, it is possible that this sequence may be responsible for the heparin-binding activity observed for $F2_{P97}$. However, an analysis of this sequence failed to identify a heparin-binding motif or a basic amino acid density that might facilitate binding.

Studies on the proteolytic cleavage of the *M. hyopneumoniae* cilium adhesin have revealed a complex processing pattern. One major processing event cleaves an N-terminal 22 kDa fragment (P22), generating the "mature" P97 adhesin (10, 37). A second major event (at position 862 for the J strain) removes a C-terminal 28 kDa fragment (P28) that separates the cilium binding epitope, R1, from R2. This event generates a central adhesin fragment that contains R1 and is approximately 66 kDa in size (P66) (10). Since both R1 and R2 are necessary for the $F2_{P97}$ fragment of the adhesin to bind heparin, the cleavage event that generates P28 is likely to impact on the ability of some cilium adhesin cleavage products to bind glycosaminoglycans. However, this cleavage event may not have a detrimental effect on the ability of P66 to bind heparin since this is likely to be conferred by the second heparin binding site found in $F1_{P97}$. The presence of two distally located heparin binding sites may allow *M. hyopneumoniae* to alter its surface architecture without a corresponding loss in heparin binding activity.

For some proteins, the ability to bind heparin has been attributed to the presence of relatively high numbers of evenly spaced basic amino acid residues rather than the presence of specific linear motifs (20, 29). The positively-charged basic residues are believed to interact with the exposed, negatively-charged sulfate groups of heparin and other glycosaminoglycans (5, 6, 20). This may also be true of the cilium adhesin regions $F1_{P97}$ and $F2_{P97}$ which are relatively rich in lysine residues. Further evidence for this type of interaction can be derived from competitive binding assays. Binding of both $F1_{P97}$ and $F2_{P97}$ to heparin was disrupted by the addition of fucoidan, a highly sulfated fucose polymer derived from marine algae, but not by chondroitin sulfate B. While not present in mammalian systems, fucoidan is useful in binding studies due to the high degree of sulfation of its carbohydrate chains and for this reason is frequently found to be a potent inhibitor of protein-heparin interactions (14, 24). The strong inhibition of heparin binding by fucoidan highlights the significance of sulfate groups in this interaction.

Mucin, another highly sulfated compound, was also found to be a powerful inhibitor in our competitive binding assays. Mucins are abundant in the mucosal secretions lining various epithelial surfaces (25). Thus, the ability of the cilium adhesin fragments to bind mucin may in itself be highly significant, since the mucosal layer is often considered the first defense against pathogens that colonize epithelial surfaces (1). Interactions with the mucous layer of the respiratory tract would likely precede binding of *M. hyopneumoniae* to cilia; therefore binding to mucins may represent an important first step in the pathogenesis of this organism. Further studies are required to characterize this interaction.

The binding affinities (K_d) of both $F1_{P97}$ (0.27 ± 0.02 μM) and $F2_{P97}$ (1.89 ± 0.33 μM) for heparin are within the range reported for other heparin-binding proteins (0.3 nM - 4 μM) such as antithrombin (2), interleukins 2 and 12 (14, 24), clusterin (26) and the mycobacterial heparin-binding haemagglutinin (29), suggesting that these interactions are of physiological significance. The ability of the *M. hyopneumoniae* cilium adhesin to bind to components of the ECM as well as to cilia indicates that this molecule plays a multi-functional role in adherence to the porcine respiratory tract. While more experiments are required to establish the precise role of the adhesin's interaction with glycosaminoglycans *in vivo*, studies of microbial pathogenesis continue to highlight the importance of binding to heparin and other sulfated polysaccharides in the establishment, maintenance and dissemination of infection (13, 21, 27, 34). Many pathogens, including *Neisseria*, *Yersinia*, and *Staphylococcus* species, exploit the widespread ability of host proteins to bind heparin. By recruiting heparin to their surfaces, these organisms acquire the ability to interact with a multitude of host molecules and circumvent the need for individual ligand receptors (13). For organisms such as *M. hyopneumoniae*, which have small genomes and therefore a limited repertoire of proteins, the ability to bind exogenous heparin may significantly increase the potential for host-parasite interactions at the site of colonization. Host molecules known to interact with heparin include ECM components, such as fibronectin and vitronectin, and cytokines and inflammatory mediators (13). Thus, the ability of *M. hyopneumoniae* to bind heparin may have important implications with respect to modulation of the host immune system and inflammatory response in addition to adherence of the pathogen to host tissues.

Acknowledgements

This work was partly funded by an ARC-Linkage grant (LP0455306) and by the McGarvie Smith Trust. We are grateful for the skilful assistance of Katrina Stewart in cloning fragments $F2_{P97}$, $F3_{P97}$ and $F4_{P97}$.

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Figure legends

Figure 1. (A) Diagrammatic representation of the cilium adhesin gene. Arrows indicate the location of primers used to amplify DNA fragments coding for $F1_{P97}$-$F4_{P97}$. Amino acid positions corresponding to the N- and C-termini of $F1_{P97}$-$F4_{P97}$ are indicated. Numbers marked with an asterisk (*) indicate positions corresponding to the cilium adhesin of *M. hyopneumoniae* strain P5722; all other numbers correspond to the cilium adhesin *M. hyopneumoniae* strain J. The $F1_{P97}$-$F4_{P97}$ adhesin fragment sizes are indicated. **(B)** Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) showing the purified, recombinant $F1_{P97}$-$F4_{P97}$ cilium adhesin fragments. Lane 1: Prestained protein molecular weight marker (MBI Fermentas, Hanover, Maryland); band sizes correspond to 118, 86, 47, 34, 26 and 19kDa, Lane 2: $F1_{P97}$, Lane 3: $F2_{P97}$, Lane 4: $F3_{P97}$, Lane 5: $F4_{P97}$. All cilium adhesin fragments run at higher molecular weights than predicted due to abnormal migration patterns.

Figure 2. Amino acid sequences of $F1_{P97}$-$F4_{P97}$. The sequences shown correspond to the *M. hyopneumoniae* strains and numbering described in Fig 1A. $F3_{P97}$ and $F4_{P97}$ are indicated by large boxes. Small boxes indicate motifs matching the heparin-binding consensus sequences. Basic residues are shaded. Tryptophan residues that represent mutagenised TGG codons are underlined. Repeat regions 1 and 2 (R1 and R2) are italicized and underscored.

Figure 3. Ligand dot blot of denatured and undenatured cilium adhesin fragments reacted with (A) biotinylated heparin and (B) amido black. All samples were analysed on

a single blot. Position 1: undenatured $F1_{P97}$; position 2: denatured $F1_{P97}$; position 3: undenatured $F2_{P97}$, position 4: denatured $F2_{P97}$, position 5: undenatured $F3_{P97}$, position 6: denatured $F3_{P97}$, position 7: undenatured $F4_{P97}$, position 8: denatured $F4_{P97}$.

Figure 4. Binding of biotinylated heparin to immobilized cilium adhesin domains $F1_{P97}$ (●), $F2_{P97}$ (○), $F3_{P97}$ (■) and $F4_{P97}$ (□). Increasing concentrations of biotinylated heparin were added until saturation was reached. Absorbances were read after 45 min incubation in the presence of substrate. Error bars indicate the standard deviation from the mean for triplicate readings. Replicate experiments resulted in similar binding curves.

Figure 5. Heparin binding curves for **(A)** $F1_{P97}$ and **(B)** $F2_{P97}$ showing total binding (●), non-specific binding (■) and specific binding (○). Total binding was determined using the standard microtitre plate assay protocol while non-specific binding was determined in the presence of a 50-fold excess of unlabelled heparin. The specific binding curves were derived by subtracting the non-specific binding data from the total binding data. Error bars indicate the standard deviation from the mean for triplicate readings.

Figure 6. Competitive binding assays showing inhibition of heparin binding to **(A)** $F1_{P97}$ and **(B)** $F2_{P97}$ by various sulfated polysaccharides: unlabelled heparin (●), fucoidan (■), mucin (○) and chondroitin sulfate B (□). Microtitre plate binding assays were performed using the standard protocol but with an excess of each inhibitor. Error bars indicate the standard deviation from the mean for triplicate readings.

Figure 1A

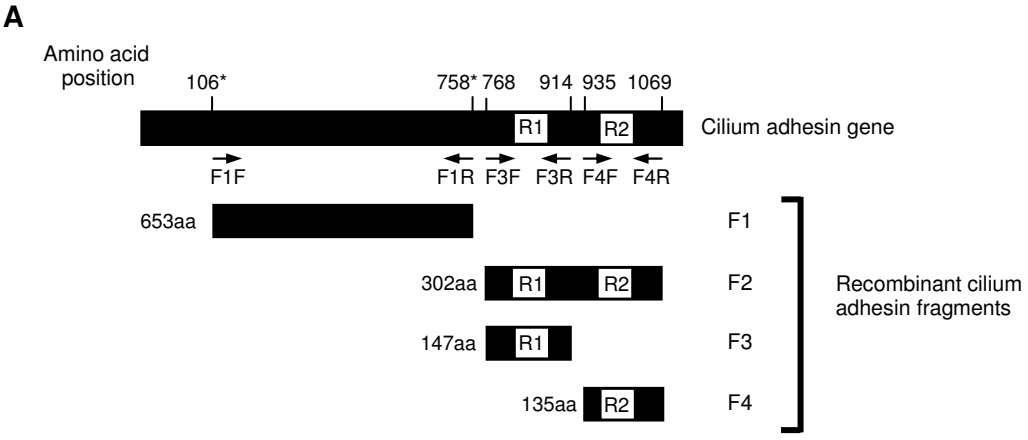

Figure 1B

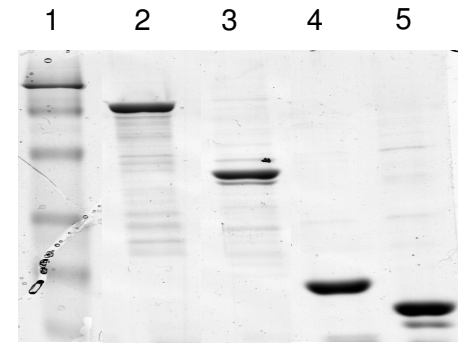

Figure 2.

F1$_{P97}$

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STLRAIDFQYDLNTARNPEDLDIKLANYFPVLKNLINRLNNAPENKLPNN
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QLAKVEVEALFKGNKNQEIYQALDGNYAYEFGAFKSVLNSWTGKIQHPEK
ADIQRFTRHLEQVKIGSNSVLNQPQTTKEQVISSLKSNNFFKNGHQVASY
FQDLLTKDKLTVLETLYDLAKKWGLETNWAQFPKGAFQYTKDIFAEADKL
KFLEWKKKDPYNQINEIHQLSFNILARNDVIKSDGFYGVLLLPQSVKTEL
EGKNEAQIFEALKKYSLIENSAFKTTILDKNLLEGTDFKTFGDFLKAFFL
KAA

F2$_{P97}$

KLDDNLQYSFEAIKKGETTKEGKREEVDKKVKELDNKIKGILPQPP*AAKP*
EAAKPVAAKPEAAKPETTKPVAAKPEAAKPVAAKPVAAKPVATNTNTNTG — **F3$_{P97}$**
FSLTNKPKEDYFPMAFSYKLEYTDENKLSLKTPEINVFLELVHQSEYEEQ
KIIKELDKTVLNLQYQFQEVKVTSEQYQKLSHPMMTE*GSPNQGKKAEGAP*
NQGKKAEGAPSQGKKAEGAPNQGKKAEGEPSQGKKAEGASNQQSTTTELT — **F4$_{P97}$**
NYLPELGKKIDEIIKKQGKNWKTEVELIEDNIAGDAKLLYFVLRDDSKSG
DP

Figure 3.

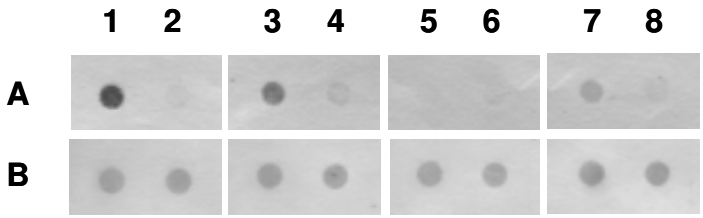

Figure 4.

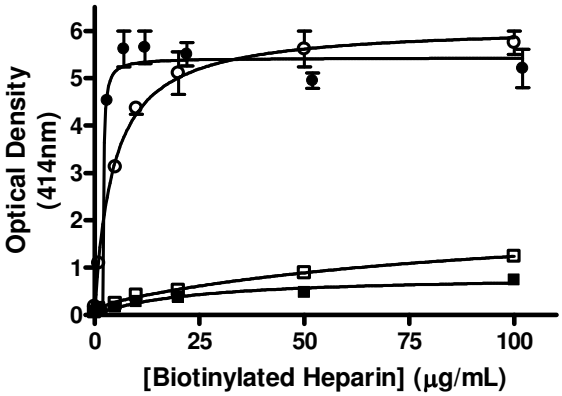
Optical Density
(414nm)
6
5
4
3
2
1
0
0
25
50
75
100
[Biotinylated Heparin] (μg/mL)

Figure 5A.

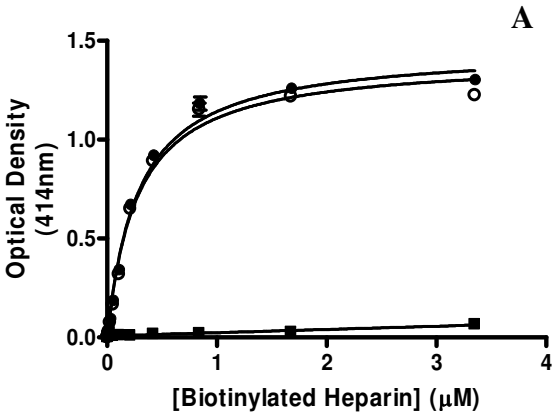

Figure 5B.

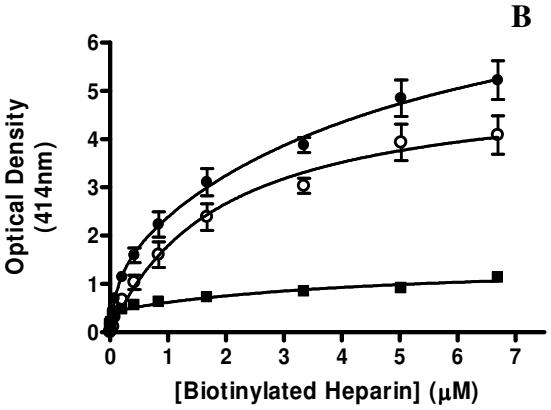

Figure 6A.

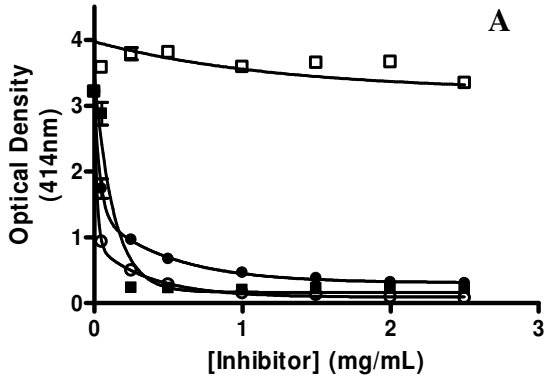

Figure 6B.